Analysis of ERBB Ligand-Induced Resistance Mechanism to Crizotinib by Primary Culture of Lung Adenocarcinoma with EML4-ALK Fusion Gene

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Introduction: Using cell line-based assays, the secretion of erythroblastic leukemia viral oncogene homologue (ERBB) ligands has been reported to contribute to resistance against crizotinib in lung cancer with the echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase fusion gene. However, it is difficult to predict the role of the ligands in each patient. Here, we report an analysis of the mechanism of resistance behind crizotinib resistance using a primary culture of cancer cells from pleural effusion of an anaplastic lymphoma kinase-positive lung cancer patient who was clinically resistant to crizotinib.

Methods: Primary cancer cells were prepared as cancer tissue-originated spheroids (CTOSs) according to previously described methods. CTOSs were maintained in StemPro medium, and a sensitivity assay was performed under growth factor-free conditions, or under stimulation with epidermal growth factor (EGF) or neuregulin-1/heregulin. The effect of treatment with crizotinib alone or a combination of crizotinib and erlotinib was examined.

Results: Cancer cells (LB53) were established to be CTOSs from a patient who was clinically resistant to crizotinib. The CTOSs were sensitive to crizotinib under growth factor-free conditions in vitro, whereas resistant under stimulation with EGF or neuregulin-1. These ligands rescued the inhibition of intracellular signaling by crizotinib. Pleural effusion from the patient also activated EGF receptor signaling to the similar extent of EGF stimulation. The resistance to crizotinib by EGF was reversed by blocking EGF receptor signaling by erlotinib in vitro.

Conclusion: Stimulation by ERBB ligands is suggested to be responsible for resistance to crizotinib in this patient. The CTOS method may enable analysis of resistance mechanism for targeted therapy in individual patients.

Key Words: Lung cancer, Anaplastic lymphoma kinase, Crizotinib resistance, Primary culture, ERBB.

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Crizotinib is an anaplastic lymphoma kinase (ALK) inhibitor that shows remarkable antitumor effects in non–small-cell lung cancer (NSCLC) patients who are positive for the echinoderm microtubule-associated protein-like 4 and ALK (*EML4-ALK*) translocation. Despite their initial response, patients with ALK-positive NSCLC treated with ALK inhibitors eventually acquire resistance to these drugs.[1] The molecular mechanisms regulating such resistance are being extensively researched. Recently, several researchers reported that the activation of erythroblastic leukemia viral oncogene homologue (ERBB) family signaling can be a mechanism by which resistance is acquired.[2–4] Using primary cultured cells from an EML4-ALK-positive tumor of a patient that was clinically resistant to crizotinib, we performed an in vitro sensitivity assay along with pathway activation/inhibition analysis.

RESULTS

Case Description

A 62-year-old woman was urgently admitted to our hospital with symptoms of productive cough and breathlessness. Computed tomography (CT) revealed multiple nodules in both lungs, left supraclavicular lymphadenopathy, bilateral pleural effusion, pericardial effusion, and multiple bone metastases, indicating T4N3M1b lung cancer. Nodule biopsy revealed lung adenocarcinoma with wild-type epidermal growth factor receptor (EGFR) and the *EML4-ALK* fusion gene (variant 1).

Figure 1 shows clinical course of the patient. Crizotinib (250 mg) was administered twice daily as the first-line treatment, but serum levels of liver enzymes became elevated. The dose of crizotinib was reduced to 200 mg twice daily, but it could not be continued because of hepatic toxicity. Finally, the patient was able to tolerate 200 mg of crizotinib once daily.

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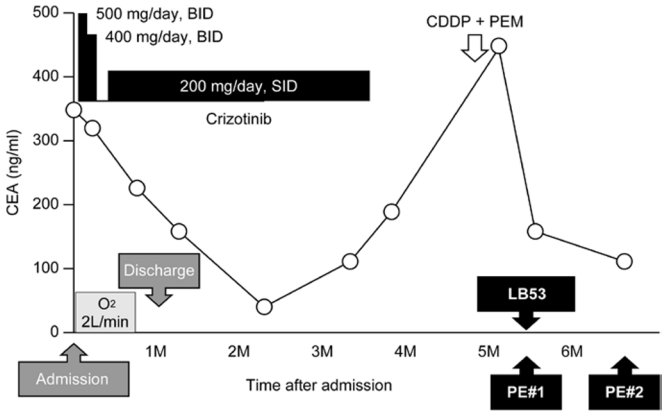

FIGURE 1. Clinical course of the patient. Crizotinib was started on her 2nd day in the hospital. The solid line indicates serum CEA value. The arrow with LB53 indicates the time when pleural effusion was obtained to establish CTOSs (LB53 cells). Time points of plural effusion sampling are indicated as PE 1 and PE 2. A month is defined as 28 days. CEA, carcinoembryonic antigen; CTOSs, cancer tissue-originated spheroids; PE, pleural effusion.

Computed tomography scans and chest radiography (Fig. 2) revealed appreciable resolution of bilateral lung shadows, and the serum value of carcinoembryonic antigen decreased (Fig. 1). The patient was discharged without oxygen support. After 3 months, however, the lung tumors started to regrow, with accumulation of pleural and pericardial effusion, despite the continued administration of crizotinib. Second-line chemotherapy of cisplatin and pemetrexed resulted in only a minor short-term response. The patient died of lung cancer 2 months after initiating the second-line chemotherapy.

Sensitivity Assay of Crizotinib Using Patient-Derived Cancer Tissue-Originated Spheroids

We successfully prepared and cultured primary cancer cells, LB53, from pleural effusion obtained after the start of the second-line chemotherapy by a recently developed primary culture method, the cancer tissue-originated spheroid (CTOS) system, which was first developed for colorectal cancer[5] and then extended to lung cancer.[6] Neither secondary mutation of the *EML4-ALK* gene nor an activating mutation of the *EGFR* gene was detected by sequencing (data not shown). A sensitivity assay was performed as previously described.[6]

LB53 cells were resistant to crizotinib in standard CTOS culture medium (StemPro hES; Invitrogen, Carlsbad, CA), whereas they were sensitive under growth factor-free conditions. Among the growth factors in the culture medium, neuregulin-1 (NRG-1) and EGF made LB53 cells more resistant to crizotinib (Fig. 3*A*, *B*). The effect was specific response to crizotinib, but not a general effect due to different culture conditions, as CTOSs from a non-EML4-ALK-translocated case showed high resistance to crizotinib in any culture condition (Supplementary Figure 1, SDC 1, http://links.lww.com/JTO/A727). In addition, rescue by ERBB ligands was not observed in *cis*-Diamineplatinum(II) dichloride (CDDP) treatment (Supplementary Figure 2, SDC 1, http://links.lww.com/JTO/A727). Pathway activation analysis revealed that phosphorylation of ALK was completely blocked by 1 μM of crizotinib in the absence or presence of EGF and NRG-1 (Fig. 3*C*). Meanwhile, EGF and NRG-1 rescued the suppression of ERK phosphorylation by crizotinib. Next, we assessed whether ERBB ligands existed in the patient's own pleural effusion. After stimulation with pleural effusion drawn at two different timings of acquired resistance, EGFR and the downstream molecule, ERK, were phosphorylated to the similar extent of EGF stimulation (Fig 4*A*). With the combined treatment of crizotinib and

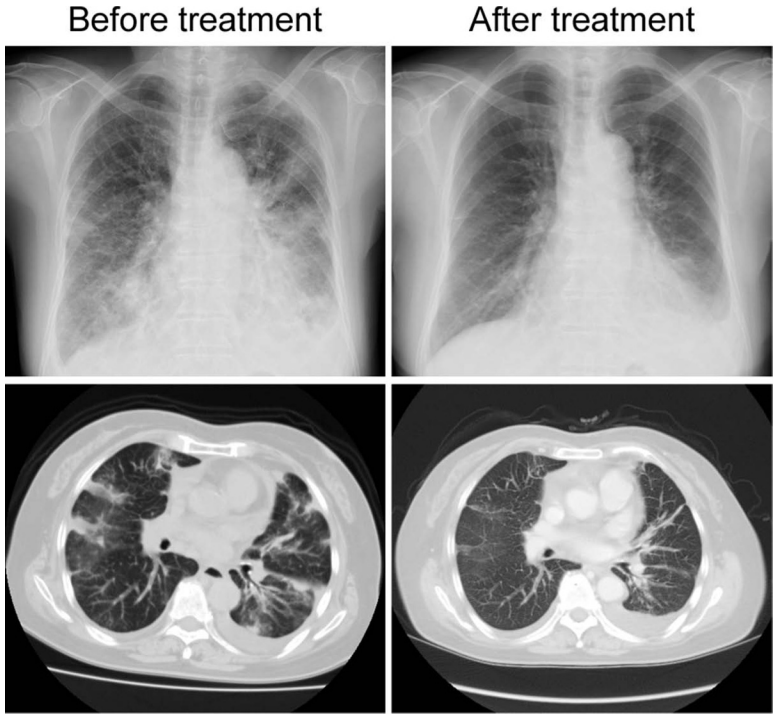

FIGURE 2. Chest radiography (upper panels) and CT (lower panels) before and after treatment with crizotinib. Chest radiography and chest CT examination after treatment were performed on the 28th day and on the 22nd day in the hospital, respectively. CT, computed tomography.

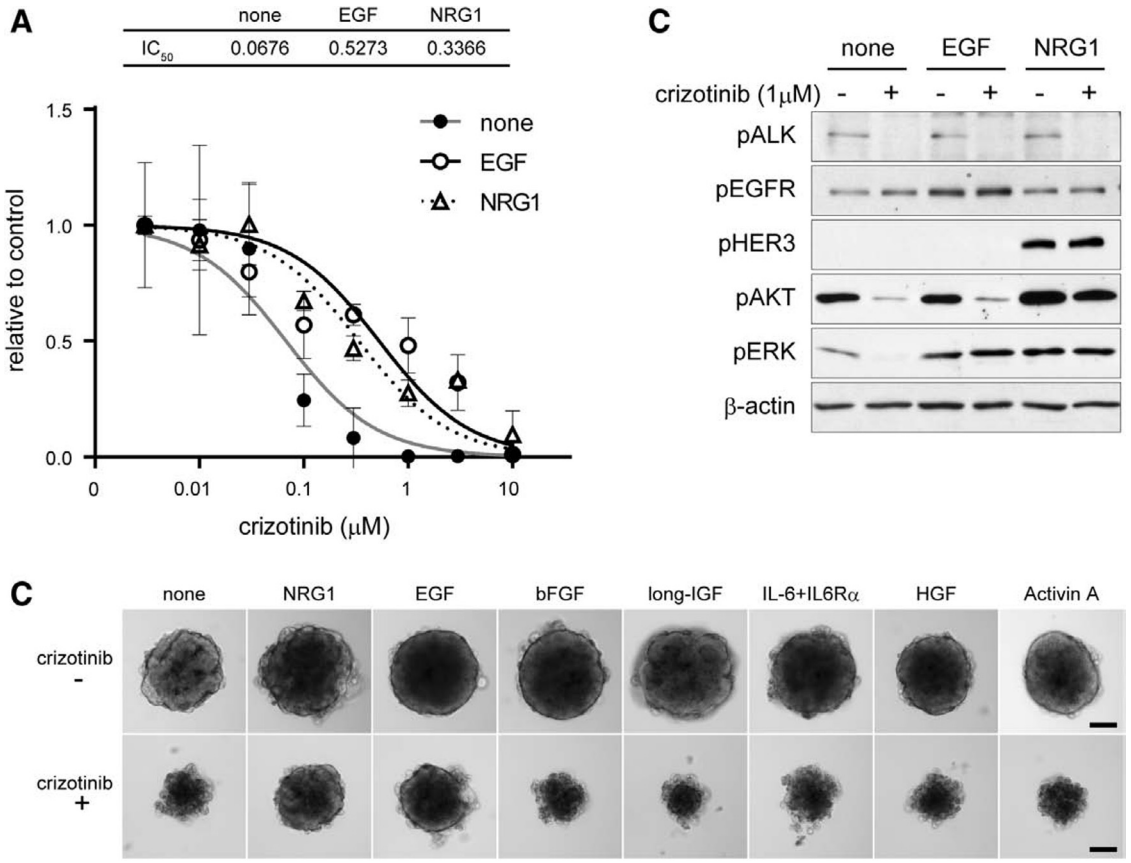

FIGURE 3. Activation of ERBB signaling by EGF or NRG-1 decreased crizotinib sensitivity. *A*, In vitro dose–response curve of crizotinib in LB53. CTOSs were cultured under growth factor-free conditions (closed circle), with 10 ng/ml of EGF (open circle), or with NRG-1 (open triangle); CTOSs were then treated with crizotinib for 7 days. Averages and standard deviation are shown. IC_{50} is listed above the graph. *B*, Representative image of CTOSs treated with 1 μM crizotinib in the presence of 10 ng/ml of NRG1, EGF, bFGF, activin A or 100 ng/ml of long-IGF1, IL-6, and IL-6Rα or 50 ng/ml of HGF for 7 days. *C*, Western blot of the ALK or ERBB signaling pathways in LB53. CTOSs were cultured under growth factor-free conditions and pulsed with 10 ng/ml of EGF or NRG-1 for 15 minutes in the presence or absence of 1 μM crizotinib. ALK, anaplastic lymphoma kinase; bFGF, basic fibroblast growth factor; CTOSs, cancer tissue-originated spheroids; EGF, epidermal growth factor; HGF, hepacyte growth factor; IC50, half maximal inhibitory concentration; NRG-1, neuregulin-1.

erlotinib (an EGFR inhibitor), LB53 cells were sensitive even in the presence of EGF (4*B*, *C*), whereas no effect was observed with erlotinib alone (Supplementary Figure 3A and B, SDC 1, http://links.lww.com/JTO/A727).

These results suggest that activation of ERBB family receptor tyrosine kinases might have been responsible for the acquired resistance to crizotinib in this patient.

DISCUSSION

Acquired resistance is the primary hurdle preventing ALK inhibitors from curing ALK-positive NSCLC. Several mechanisms of resistance have been proposed, including secondary resistant mutations, amplification of the *ALK* fusion gene, and activation of other receptor tyrosine kinases.[7] Among them, EGFR activation by secretion of EGFR ligands amphiregulin and EGF, and ERBB3 ligand NRG-1 by cancer cells has been shown to mediate crizotinib resistance by cell line-based analyses.[2,4,7]

These mechanisms of resistance can overlap. Therefore, a snapshot analysis using biopsy samples, such as assessing phosphorylation of EGFR, is not sufficient to predict the response of a patient to a combination therapy such as ALK inhibitors plus EGFR tyrosine kinase inhibitors. Direct assessment of sensitivity to the drugs or their combination is necessary, especially in cases of ligand-dependent resistance, as in this report.

We tested the above-mentioned mechanisms of resistance using primary culture samples. The CTOS method has high efficiency and purity for a primary culture of cancer cells and can be applied to sensitivity assay and pathway activation analysis.[5,6,8] In lung cancer, the success rate of CTOS preparation is 80.0% from surgical specimens and 44.4% from pleural effusion; the latter totally depends on the contents of cancer cells in the withdrawn effusion.[6] Although CTOS preparation from tiny biopsy specimens still remains challenging, CTOS can be established in a few days when sufficient amount of tumor cells are obtained. Once established, the CTOS sensitivity assay and its pathway activation analysis might enable the selection of suitable patients for combination therapy of targeted drugs. Especially, assessing the concentration of each ligand in culture medium might help

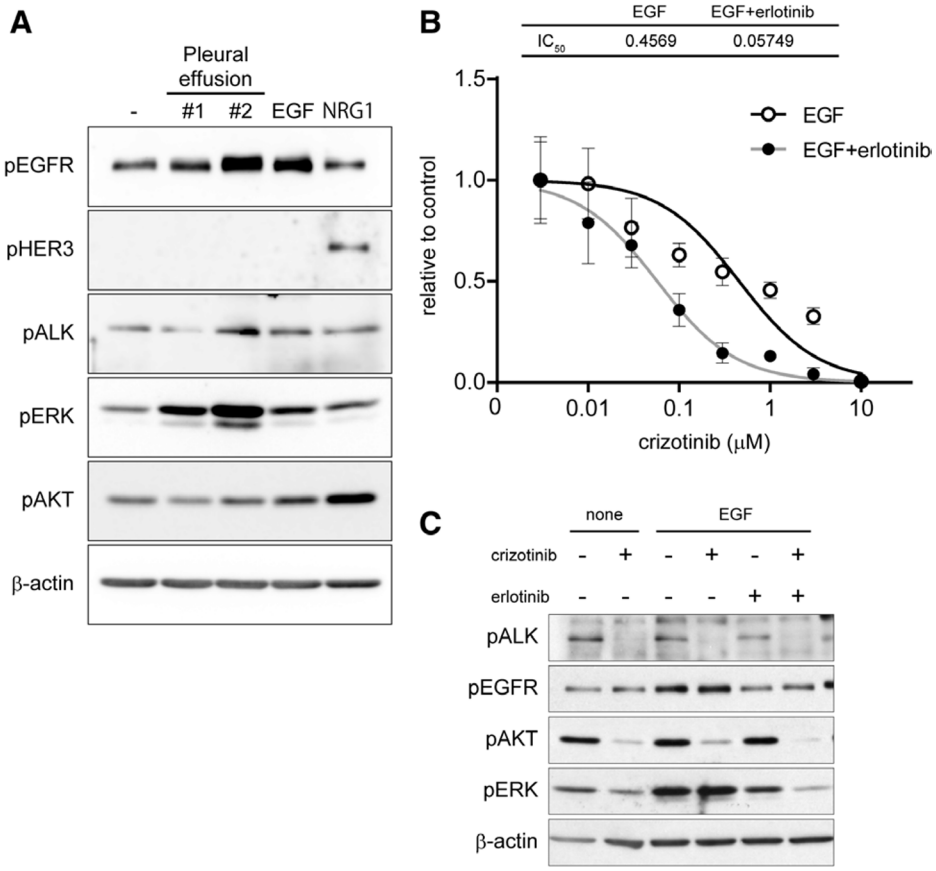

FIGURE 4. Erlotinib restored crizotinib sensitivity in the presence of EGF. *A*, Western blot of ALK and ERBB signaling pathways in LB53. CTOSs were cultured under growth factor-free conditions over night, and then stimulation for 15 minutes was performed with patient's own pleural effusion, or with the medium containing 10 ng/ml of EGF or NRG-1. Pleural effusion drawn at two different time points was examined (Pleural effusion 1 and 2). *B*, In vitro dose–response curve of crizotinib in LB53 cells. CTOSs were cultured in medium containing 10 ng/ml of EGF with (closed circle) or without (open circle) 1 μM of erlotinib. Average and standard deviation are shown. IC_{50} is listed above the graph. *C*, Western blot of ALK or the ERBB signaling pathway in LB53. CTOSs were cultured under growth factor-free conditions and pulsed with 10 ng/ml EGF for 15 minutes in the presence or absence of 1 μM crizotinib and/or 1 μM erlotinib. EGF, epidermal growth factor; NRG-1, neuregulin-1; CTOSs, cancer tissue-originated spheroids; ALK, anaplastic lymphoma kinase.

determine which specific ERBB family receptor signals should be blocked to overcome crizotinib resistance.

This is the first report regarding preparation and culturing CTOSs of ALK-positive lung cancer from the pleural effusion of a patient whose disease was clinically resistant to crizotinib. Using the CTOS system, we suggested that activation of ERBB family receptor tyrosine kinase has facilitated a mechanism of resistance to crizotinib in this patient. Although these analyses did not contribute to her treatment itself, our results suggest that the sensitivity assay with primary cancer cells using the CTOS method may contribute to the determination of treatment strategy in the era of molecular target drugs.

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